JUL 18 2001
PATENT & TRADEMARK OFFICE
TC 1600/2900

RECEIVED
JUL 20 2001
TECH CENTER 1600/2900

have available alternative, non-surgical diagnostic methods capable of detecting early stage breast disease, such as cancer.

In contrast to the standard translation of mRNA into polypeptide, rare errors in translation have been reported, termed translational frameshifting, that result in contiguous polypeptide encoded by two different reading frames P.J. Farabaugh, Annual Rev. Genet. 30: 507-528 (1996).

Summary of the Invention

The present invention provides a method of detecting a target BS322 polynucleotide in a test sample which comprises contacting the test sample with at least one BS322-specific polynucleotide and detecting the presence of the target BS322 polynucleotide in the test sample. The BS322-specific polynucleotide has at least 50% identity with a polynucleotide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9 (SEQ ID NOS 1-9), and fragments or complements thereof. Also, the BS322-specific polynucleotide may be attached to a solid phase prior to performing the method.

The present invention also provides a method for detecting BS322 mRNA in a test sample, that comprises performing reverse transcription (RT) with at least one primer in order to produce cDNA, amplifying the cDNA so obtained using BS322 oligonucleotides as sense and antisense primers to obtain BS322 amplicon, and detecting the presence of the BS322 amplicon as an indication of the presence of BS322 mRNA in the test sample, wherein the BS322 oligonucleotides have at least 50% identity with a sequence selected from the group consisting of SEQ ID NOS 1-9, and fragments or complements thereof. Amplification can be performed by the polymerase chain reaction. Also, the test sample can be reacted with a solid phase prior to performing the method, prior to amplification or prior to detection. This reaction can be a direct or an indirect reaction. Further, the detection step can comprise utilizing a detectable label capable of generating a measurable signal. The detectable label can be attached to a solid phase.

The present invention further provides a method of detecting a target BS322 polynucleotide in a test sample suspected of containing target BS322 polynucleotides,

that comprises (a) contacting the test sample with at least one BS322 oligonucleotide as a sense primer and at least one BS322 oligonucleotide as an anti-sense primer, and amplifying same to obtain a first stage reaction product; (b) contacting the first stage reaction product with at least one other BS322 oligonucleotide to obtain a second stage reaction product, with the proviso that the other BS322 oligonucleotide is located 3' to the BS322 oligonucleotides utilized in step (a) and is complementary to the first stage reaction product; and (c) detecting the second stage reaction product as an indication of the presence of a target BS322 polynucleotide in the test sample. The BS322 oligonucleotides selected as reagents in the method have at least 50% identity with a sequence selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof. Amplification may be performed by the polymerase chain reaction. The test sample can be reacted either directly or indirectly with a solid phase prior to performing the method, or prior to amplification, or prior to detection. The detection step also comprises utilizing a detectable label capable of generating a measurable signal; further, the detectable label can be attached to a solid phase.

Test kits useful for detecting target BS322 polynucleotide in a test sample are also provided which comprise a container containing at least one BS322 specific polynucleotide selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof. These test kits further comprise containers with tools useful for collecting test samples (such as, for example, blood, urine, saliva and stool). Such tools include lancets and absorbent paper or cloth for collecting and stabilizing blood; swabs for collecting and stabilizing saliva; and cups for collecting and stabilizing urine or stool samples. Collection materials, such as papers, cloths, swabs, cups, and the like, may optionally be treated to avoid denaturation or irreversible adsorption of the sample. The collection materials also may be treated with or contain preservatives, stabilizers or antimicrobial agents to help maintain the integrity of the specimens.

The present invention also provides a purified polynucleotide or fragment thereof derived from a BS322 gene. The purified polynucleotide is capable of selectively hybridizing to the nucleic acid of the BS322 gene, or a complement thereof. The polynucleotide has at least 50% identity with a polynucleotide selected from the group consisting of SEQ ID NO:1-9, and fragments or

complements thereof. Further, the purified polynucleotide can be produced by recombinant and/or synthetic techniques. The purified recombinant polynucleotide can be contained within a recombinant vector. The invention further comprises a host cell transfected with the recombinant vector.

The present invention further provides a recombinant expression system comprising a nucleic acid sequence that includes an open reading frame derived from BS322. The nucleic acid sequence has at least 50% identity with a sequence selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof. The nucleic acid sequence is operably linked to a control sequence compatible with a desired host. Also provided is a cell transfected with this recombinant expression system.

The present invention also provides a polypeptide encoded by BS322. The polypeptide can be produced by recombinant technology, provided in purified form, or produced by synthetic techniques. The polypeptide comprises an amino acid sequence which has at least 50% identity with an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof.

Also provided is a specific binding molecule, such as an antibody, which specifically binds to at least one BS322 epitope. The antibody can be a polyclonal or monoclonal antibody. The epitope is derived from an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. Assay kits for determining the presence of BS322 antigen or anti-BS322 antibody in a test sample are also included. In one embodiment, the assay kits comprise a container containing at least one BS322 polypeptide having at least 50% identity with an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. Further, the test kit can comprise a container with tools useful for collecting test samples (such as blood, urine, saliva, and stool). Such tools include lancets and absorbent paper or cloth for collecting and stabilizing blood; swabs for collecting and stabilizing saliva; and cups for collecting and stabilizing urine or stool samples. Collection materials

such as papers, cloths, swabs, cups, and the like, may optionally be treated to avoid denaturation or irreversible adsorption of the sample. These collection materials also may be treated with or contain preservatives, stabilizers or antimicrobial agents to help maintain the integrity of the specimens. Also, the polypeptide can be attached to a solid phase.

In another embodiment of the invention, specific binding molecules such as antibodies or fragments thereof against the BS322 antigen can be used to detect or image localization of the antigen in a patient for the purpose of detecting or diagnosing a disease or condition. Such antibodies can be polyclonal or monoclonal, or made by molecular biology techniques, and can be labeled with a variety of detectable labels, including but not limited to radioisotopes and paramagnetic metals. Furthermore, antibodies or fragments thereof, whether monoclonal, polyclonal, or made by molecular biology techniques, can be used as therapeutic agents for the treatment of diseases characterized by expression of the BS322 antigen. In the case of therapeutic applications, the antibody may be used without derivitization, or it may be derivitized with a cytotoxic agent such as a radioisotope, enzyme, toxin, drug, prodrug, or the like.

Another assay kit for determining the presence of BS322 antigen or anti-BS322 antibody in a test sample comprises a container containing a specific binding molecule, such as an antibody, which specifically binds to a BS322 antigen, wherein the BS322 antigen comprises at least one BS322-encoded epitope. The BS322 antigen has at least about 50% sequence identity to a sequence of a BS322-encoded antigen selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. These test kits can further comprise containers with tools useful for collecting test samples (such as blood, urine, saliva, and stool). Such tools include lancets and absorbent paper or cloth for collecting and stabilizing blood; swabs for collecting and stabilizing saliva; cups for collecting and stabilizing urine or stool samples. Collection materials, such as papers, cloths, swabs, cups and the like, may optionally be treated to avoid denaturation or irreversible adsorption of the sample. These collection materials also may be treated with, or contain, preservatives, stabilizers or antimicrobial agents to help maintain the integrity of the specimens. The antibody can be attached to a solid phase.

A method for producing a polypeptide which contains at least one epitope of BS322 is provided, which method comprises incubating host cells transfected with an expression vector. This vector comprises a polynucleotide sequence encoding a polypeptide, wherein the polypeptide comprises an amino acid sequence having at least 50% identity with a BS322 amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof.

A method for detecting BS322 antigen in a test sample suspected of containing BS322 antigen also is provided. The method comprises contacting the test sample with an antibody or fragment thereof which specifically binds to at least one epitope of BS322 antigen, for a time and under conditions sufficient for the formation of antibody/antigen complexes; and detecting the presence of such complexes containing the antibody as an indication of the presence of BS322 antigen in the test sample. The antibody can be attached to a solid phase and may be either a monoclonal or polyclonal antibody. Furthermore, the antibody specifically binds to at least one BS322 antigen selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof.

Another method is provided which detects antibodies which specifically bind to BS322 antigen in a test sample suspected of containing these antibodies. The method comprises contacting the test sample with a polypeptide which contains at least one BS322 epitope, wherein the BS322 epitope comprises an amino acid sequence having at least 50% identity with an amino acid sequence encoded by a BS322 polynucleotide, or a fragment thereof. Contacting is performed for a time and under conditions sufficient to allow antigen/antibody complexes to form. The method further entails detecting complexes which contain the polypeptide. The polypeptide can be attached to a solid phase. Further, the polypeptide can be a recombinant protein or a synthetic peptide having at least 50% identity with an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof.

The present invention provides a cell transfected with a BS322 nucleic acid sequence that encodes at least one epitope of a BS322 antigen, or fragment thereof.

The nucleic acid sequence is selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof.

A method for producing antibodies to BS322 antigen also is provided, which method comprises administering to an individual an isolated immunogenic polypeptide or fragment thereof, wherein the isolated immunogenic polypeptide comprises at least one BS322 epitope. The immunogenic polypeptide is administered in an amount sufficient to produce an immune response. The isolated, immunogenic polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof.

Another method for producing antibodies which specifically bind to BS322 antigen is disclosed, which method comprises administering to an individual a plasmid comprising a nucleic acid sequence which encodes at least one BS322 epitope derived from an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. The plasmid is administered in an amount such that the plasmid is taken up by cells in the individual and expressed at levels sufficient to produce an immune response.

Also provided is a composition of matter that comprises a BS322 polynucleotide of at least about 10-12 nucleotides having at least 50% identity with a polynucleotide selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof. The BS322 polynucleotide encodes an amino acid sequence having at least one BS322 epitope. Another composition of matter provided by the present invention comprises a polypeptide with at least one BS322 epitope of about 8-10 amino acids. The polypeptide comprises an amino acid sequence having at least 50% identity with an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. Also provided is a gene, or fragment thereof, coding for a BS322 polypeptide which has at least 50% identity with SEQ ID NO:24 or SEQ ID NO:25, and a gene, or a fragment thereof comprising DNA having at least 50% identity with SEQ ID NO:8 and SEQ ID NO:9.

Brief Description of the Drawings

Figures 1A-1E show the nucleotide alignment of clones 4304443H1 (SEQ ID NO:1), 3040232H1 (SEQ ID NO:2), 3790941H1 (SEQ ID NO:3), 3424294H1 (SEQ ID NO:4), 2741038H1 (SEQ ID NO:5), 4302934H1 (SEQ ID NO:6), 158545H1 (SEQ ID NO:7), the full-length sequence of clone 4304443H1 [designated as 4304443inh (SEQ ID NO:8)], and the consensus sequence (SEQ ID NO:9) derived therefrom.

Figure 2 shows the contig map depicting the formation of the consensus nucleotide sequence (SEQ ID NO:9) from the nucleotide alignment of overlapping clones 4304443H1 (SEQ ID NO:1), 3040232H1 (SEQ ID NO:2), 3790941H1 (SEQ ID NO:3), 3424294H1 (SEQ ID NO:4), 2741038H1 (SEQ ID NO:5), 4302934H1 (SEQ ID NO:6), 158545H1 (SEQ ID NO:7), 4304443inh (SEQ ID NO:8).

Detailed Description of the Invention

The present invention provides a gene, or a fragment thereof, which codes for a BS322 polypeptide having at least about 50% identity with SEQ ID NO:24 or SEQ ID NO:25. The present invention further encompasses a BS322 gene, or a fragment thereof, comprising DNA which has at least about 50% identity with SEQ ID NO:8 or SEQ ID NO:9.

The present invention also provides methods for assaying a test sample for products of a breast tissue gene designated as BS322, which comprises making cDNA from mRNA in the test sample, and detecting the cDNA as an indication of the presence of breast tissue gene BS322. The method may include an amplification step, wherein one or more portions of the mRNA from BS322 corresponding to the gene or fragments thereof, is amplified. Methods also are provided for assaying for the translation products of BS322. Test samples which may be assayed by the methods provided herein include tissues, cells, body fluids and secretions. The present invention also provides reagents such as oligonucleotide primers and polypeptides which are useful in performing these methods.

Portions of the nucleic acid sequences disclosed herein are useful as primers for the reverse transcription of RNA or for the amplification of cDNA; or as probes to

the polynucleotide encodes a polypeptide fragment, the polynucleotide fragment length will be the number of base pairs necessary to encode the polypeptide fragment of interest. Representative polypeptide fragment lengths are given below. Polynucleotides encoding these polypeptide fragments are therefore encompassed by the present invention.

The term "primer" denotes a specific oligonucleotide sequence which is complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence. A primer serves as an initiation point for nucleotide polymerization catalyzed by DNA polymerase, RNA polymerase, or reverse transcriptase.

The term "probe" denotes a defined nucleic acid segment (or nucleotide analog segment, e.g., PNA as defined hereinbelow) which can be used to identify a specific polynucleotide present in samples bearing the complementary sequence.

"Encoded by" refers to a nucleic acid sequence which codes for a polypeptide sequence, wherein the polypeptide sequence or a portion thereof contains an amino acid sequence of at least 3 to 5 amino acids, more preferably at least 8 to 10 amino acids, and even more preferably at least 15 to 20 amino acids from a polypeptide encoded by the nucleic acid sequence. Also encompassed are polypeptide sequences which are immunologically identifiable with a polypeptide encoded by the sequence. Thus, a "polypeptide," "protein," or "amino acid" sequence has at least about 50% identity, preferably about 60% identity, more preferably about 75-85% identity, and most preferably about 90-95% or more identity with a BS322 amino acid sequence. Further, the BS322 "polypeptide," "protein," or "amino acid" sequence may have at least about 60% similarity, preferably at least about 75% similarity, more preferably about 85% similarity, and most preferably about 95% or more similarity to a polypeptide or amino acid sequence of BS322. This amino acid sequence can be selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof.

A "recombinant polypeptide," "recombinant protein," or "a polypeptide produced by recombinant techniques," which terms may be used interchangeably herein, describes a polypeptide which by virtue of its origin or manipulation is not

The following is the general procedure for the isolation and analysis of cDNA clones. In a particular embodiment disclosed herein, mRNA is isolated from breast tissue and used to generate the cDNA library. Breast tissue is obtained from patients by surgical resection and is classified as tumor or non-tumor tissue by a pathologist.

The cDNA inserts from random isolates of the breast tissue libraries are sequenced in part, analyzed in detail as set forth in the Examples, and are disclosed in the Sequence Listing as SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7. Also analyzed in detail as set forth in the Examples, and disclosed in the Sequence Listing, is the full-length sequence of clone 4304443H1 [referred to herein as 4304443inh (SEQ ID NO:8)]. The consensus sequence of these inserts is presented as SEQ ID NO:9. These polynucleotides may contain an entire open reading frame with or without associated regulatory sequences for a particular gene, or they may encode only a portion of the gene of interest. This is attributed to the fact that many genes are several hundred and sometimes several thousand bases in length and, with current technology, cannot be cloned in their entirety because of vector limitations, incomplete reverse transcription of the first strand, or incomplete replication of the second strand. Contiguous, secondary clones containing additional nucleotide sequences may be obtained using a variety of methods known to those of skill in the art.

Methods for DNA sequencing are well known in the art. Conventional enzymatic methods employ DNA polymerase, Klenow fragment, Sequenase (US Biochemical Corp, Cleveland, OH) or Taq polymerase to extend DNA chains from an oligonucleotide primer annealed to the DNA template of interest. Methods have been developed for the use of both single-stranded and double-stranded templates. The chain termination reaction products may be electrophoresed on urea/polyacrylamide gels and detected either by autoradiography (for radionucleotide labeled precursors) or by fluorescence (for fluorescent-labeled precursors). Recent improvements in mechanized reaction preparation, sequencing and analysis using the fluorescent detection method have permitted expansion in the number of sequences that can be determined per day using machines such as the Applied Biosystems 377 DNA Sequencers (Applied Biosystems, Foster City, CA).

The monoclonal antibodies or fragments thereof can be provided individually to detect BS322 antigens. Combinations of the monoclonal antibodies (and fragments thereof) provided herein also may be used together as components in a mixture or "cocktail" of at least one BS322 antibody of the invention, along with antibodies which specifically bind to other BS322 regions, each antibody having different binding specificities. Thus, this cocktail can include the monoclonal antibodies of the invention which are directed to BS322 polypeptides disclosed herein and other monoclonal antibodies specific to other antigenic determinants of BS322 antigens or other related proteins.

The polyclonal antibody or fragment thereof which can be used in the assay formats should specifically bind to a BS322 polypeptide or other BS322 polypeptides additionally used in the assay. The polyclonal antibody used preferably is of mammalian origin such as, human, goat, rabbit or sheep polyclonal antibody that binds BS322 polypeptide. Most preferably, the polyclonal antibody is of rabbit origin. The polyclonal antibodies used in the assays can be used either alone or as a cocktail of polyclonal antibodies. Since the cocktails used in the assay formats are comprised of either monoclonal antibodies or polyclonal antibodies having different binding specificity to BS322 polypeptides, they are useful for the detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating, or determining the predisposition to, diseases and conditions of the breast, such as breast cancer.

It is contemplated and within the scope of the present invention that BS322 antigen may be detectable in assays by use of a recombinant antigen as well as by use of a synthetic peptide or purified peptide, which peptide comprises an amino acid sequence of BS322. The amino acid sequence of such a polypeptide is selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. It also is within the scope of the present invention that different synthetic, recombinant or purified peptides, identifying different epitopes of BS322, can be used in combination in an assay for the detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating, or determining the predisposition to diseases and conditions of the breast, such as breast cancer. In this case, all of these peptides can be coated onto one solid phase; or each separate

the expression systems as disclosed herein. For example, in this assay system, BS322 antigen can be the first analyte. Such assay systems are described in greater detail in EP Publication No. 0473065.

In yet other assay formats, the polypeptides disclosed herein may be utilized to detect the presence of antibody against BS322 antigen in test samples. For example, a test sample is incubated with a solid phase to which at least one polypeptide such as a recombinant protein or synthetic peptide has been attached. The polypeptide is selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. These are reacted for a time and under conditions sufficient to form antigen/antibody complexes. Following incubation, the antigen/antibody complex is detected. Indicator reagents may be used to facilitate detection, depending upon the assay system chosen. In another assay format, a test sample is contacted with a solid phase to which a recombinant protein produced as described herein is attached, and also is contacted with a monoclonal or polyclonal antibody specific for the protein, which preferably has been labeled with an indicator reagent. After incubation for a time and under conditions sufficient for antibody/antigen complexes to form, the solid phase is separated from the free phase, and the label is detected in either the solid or free phase as an indication of the presence of antibody against BS322 antigen. Other assay formats utilizing the recombinant antigens disclosed herein are contemplated. These include contacting a test sample with a solid phase to which at least one antigen from a first source has been attached, incubating the solid phase and test sample for a time and under conditions sufficient to form antigen/antibody complexes, and then contacting the solid phase with a labeled antigen, which antigen is derived from a second source different from the first source. For example, a recombinant protein derived from a first source such as E. coli is used as a capture antigen on a solid phase, a test sample is added to the so-prepared solid phase, and following standard incubation and washing steps as deemed or required, a recombinant protein derived from a different source (i.e., non-E. coli) is utilized as a part of an indicator reagent which subsequently is detected. Likewise, combinations of a recombinant antigen on a solid phase and synthetic peptide in the indicator phase also are possible. Any assay format that utilizes an antigen specific for BS322 produced or derived from a first source as the capture antigen and an antigen specific

adsorption to a test piece that comprises a solid phase of a plastic or metal surface, following methods known to those of ordinary skill in the art. Or, covalent attachment of a specific binding partner (analyte specific substance) to a test piece which test piece comprises a solid phase of derivatized plastic, metal, silicon, or glass may be utilized. Covalent attachment methods are known to those skilled in the art and include a variety of means to irreversibly link specific binding partners to the test piece. If the test piece is silicon or glass, the surface must be activated prior to attaching the specific binding partner. Also, polyelectrolyte interactions may be used to immobilize a specific binding partner on a surface of a test piece by using techniques and chemistries. The preferred method of attachment is by covalent means. Following attachment of a specific binding member, the surface may be further treated with materials such as serum, proteins, or other blocking agents to minimize non-specific binding. The surface also may be scanned either at the site of manufacture or point of use to verify its suitability for assay purposes. The scanning process is not anticipated to alter the specific binding properties of the test piece.

While the present invention discloses the preference for the use of solid phases, it is contemplated that the reagents such as antibodies, proteins and peptides of the present invention can be utilized in non-solid phase assay systems. These assay systems are known to those skilled in the art, and are considered to be within the scope of the present invention.

It is contemplated that the reagent employed for the assay can be provided in the form of a test kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a probe, primer, monoclonal antibody or a cocktail of monoclonal antibodies, or a polypeptide (e.g. recombinantly, synthetically produced or purified) employed in the assay. The polypeptide is selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, and fragments thereof. Other components such as buffers, controls and the like, known to those of ordinary skill in art, may be included in such test kits. It also is contemplated to provide test kits which have means for collecting test samples comprising accessible body fluids, e.g., blood, urine, saliva and stool. Such tools useful for collection ("collection materials") include lancets and absorbent paper or cloth for collecting and stabilizing blood; swabs for collecting and stabilizing saliva; cups for

## EXAMPLES

### Example 1: Identification of Breast Tissue Library BS322 Gene-Specific Clones

#### A. Library Comparison of Expressed Sequence Tags (EST's) or Transcript

Images. Partial sequences of cDNA clone inserts, so-called "expressed sequence tags" (EST's), were derived from cDNA libraries made from breast tumor tissues, breast non-tumor tissues and numerous other tissues, both tumor and non-tumor and entered into a database (LIFESEQ™ database, available from Incyte Pharmaceuticals, Palo Alto, CA) as gene transcript images. See International Publication No. WO 95/20681. (A transcript image is a listing of the number of EST's for each of the represented genes in a given tissue library. EST's sharing regions of mutual sequence overlap are classified into clusters. A cluster is assigned a clone number from a representative 5' EST. Often, a cluster of interest can be extended by comparing its consensus sequence with sequences of other EST's which did not meet the criteria for automated clustering. The alignment of all available clusters and single EST's represent a contig from which a consensus sequence is derived.) The transcript images then were evaluated to identify EST sequences that were representative primarily of the breast tissue libraries. These target clones then were ranked according to their abundance (occurrence) in the target libraries and their absence from background libraries. Higher abundance clones with low background occurrence were given higher study priority. EST's corresponding to the consensus sequence of BS322 were found in 23.2% (10 of 43) of breast tissue libraries. EST's corresponding to the consensus sequence, SEQ ID NO:9 (or fragments thereof) were found in only 0.1% (1 of 762) of the other, non-breast, libraries of the data base. Therefore, the consensus sequence or fragment thereof was found more than 177 times more often in breast than non-breast tissues. Overlapping clones 4304443H1 (SEQ ID NO:1), 3040232H1 (SEQ ID NO:2), 3790941H1 (SEQ ID NO:3), 3424294H1 (SEQ ID NO:4), 2741038H1 (SEQ ID NO:5), 4302934H1 (SEQ ID NO:6), 158545H1 (SEQ ID NO:7), respectively, were identified for further study. These represented the minimum number of clones that (along with the full-length sequence of clone 4304443H1 [designated as 4304443inh (SEQ ID NO:8)] were needed to form the contig

and from which the consensus sequence provided herein (SEQ ID NO:9) was derived.

B. Generation of a Consensus Sequence. The nucleotide sequences of clones 4304443H1 (SEQ ID NO:1), 3040232H1 (SEQ ID NO:2), 3790941H1 (SEQ ID NO:3), 3424294H1 (SEQ ID NO:4), 2741038H1 (SEQ ID NO:5), 4302934H1 (SEQ ID NO:6), 158545H1 (SEQ ID NO:7) and the full-length sequence of clone 4304443H1 [designated as 4304443inh (SEQ ID NO:8)] were entered in the Sequencher™ Program (available from Gene Codes Corporation, Ann Arbor, MI) in order to generate a nucleotide alignment (contig map) and then generate their consensus sequence (SEQ ID NO:9). Figures 1A-1E show the nucleotide sequence alignment of these clones and their resultant nucleotide consensus sequence (SEQ ID NO:9). Figure 2 presents the contig map depicting the clones 4304443H1 (SEQ ID NO:1), 3040232H1 (SEQ ID NO:2), 3790941H1 (SEQ ID NO:3), 3424294H1 (SEQ ID NO:4), 2741038H1 (SEQ ID NO:5), 4302934H1 (SEQ ID NO:6), 158545H1 (SEQ ID NO:7), and the full-length sequence of clone 4304443H1 [designated as 4304443inh (SEQ ID NO:8)] that form overlapping regions of the BS322 gene and the resultant consensus nucleotide sequence (SEQ ID NO:9) of these clones in a graphic display. Following this, a three-frame translation was performed on the consensus sequence (SEQ ID NO:9). The third forward frame was found to have an open reading frame encoding a 398-residue amino acid sequence that is presented as SEQ ID NO:24. The open reading frame corresponds to nucleotides 57–1250 of SEQ ID NO:9. A second coding region was found in the second forward reading frame and overlaps the first. This open reading frame (corresponding to nucleotides 1171-2122 of SEQ ID NO:9) encodes a 317-residue amino acid sequence which is presented as SEQ ID NO:25. It is known that rare errors in translation, termed translational frameshifting, occur that allow the ribosome to translate two partially overlapping reading frames as a single polypeptide. I.P. Ivanov et al. RNA 4(10):1230-1238 (1998); and P.J. Farabaugh Annu Rev Genet 30:507-528 (1996). Thus, it is within the scope of this invention that these two partially overlapping reading frames may be translated as such a single polypeptide.

Example 2: Sequencing of BS322 EST-Specific Clones

The DNA sequence of clone 4304443H1 of the BS322 gene contig was determined (SEQ ID NO:8) using dideoxy termination sequencing with dye terminators following known methods [F. Sanger et al., PNAS U.S.A. 74:5463 (1977)].

Because vectors such as pSPORT1 (Life Technologies, Gaithersburg, MD) and pINCY (available from Incyte Pharmaceuticals, Inc., Palo Alto, CA) contain universal priming sites just adjacent to the 3' and 5' ligation junctions of the inserts, the inserts were sequenced in both directions using universal primers, SEQ ID NO:12 and SEQ ID NO:13 ( New England Biolabs, Beverly, MA and Applied Biosystems Inc, Foster City, CA, respectively). The sequencing reactions were run on a polyacrylamide denaturing gel, and the sequences were determined by an Applied Biosystems 377 Sequencer (available from Applied Biosystems, Foster City, CA). Additional sequencing primers, SEQ ID NO:14-23 were designed from sequence information of the consensus sequence, SEQ ID NO:9. These primers then were used to determine the remaining DNA sequence of the cloned insert from each DNA strand, as previously described.

Example 3: Nucleic Acid

A. RNA Extraction from Tissue. Total RNA is isolated from breast tissues and from non-breast tissues. Various methods are utilized, including but not limited to the lithium chloride/urea technique, known in the art and described by Kato et al., (J. Virol. 61:2182-2191, 1987), and TRIzol™ (Gibco-BRL, Grand Island, NY).

Briefly, tissue is placed in a sterile conical tube on ice and 10-15 volumes of 3 M LiCl, 6 M urea, 5 mM EDTA, 0.1 M -mercaptoethanol, 50 mM Tris-HCl (pH 7.5) are added. The tissue is homogenized with a Polytron® homogenizer (Brinkman Instruments, Inc., Westbury, NY) for 30-50 sec on ice. The solution is transferred to a 15 ml plastic centrifuge tube and placed overnight at -20°C. The tube is centrifuged for 90 min at 9,000 x g at 0-4°C and the supernatant is immediately decanted. Ten ml of 3 M LiCl are added and the tube is vortexed for 5 sec. The tube is centrifuged for 45 min at 11,000 x g at 0-4°C. The decanting, resuspension in LiCl, and centrifugation is repeated and the final pellet is air dried and suspended in 2 ml of 1

C. RNase Digestion. RNA that is not hybridized to probe is removed from the reaction as per the Direct Protect™ protocol using a solution of RNase A and RNase T1 for 30 min at 37°C, followed by removal of RNase by Proteinase K digestion in the presence of sodium sarcosyl. Hybridized fragments protected from digestion are then precipitated by the addition of an equal volume of isopropanol and placed at -70°C for 3 hr. The precipitates are collected by centrifugation at 12,000 x g for 20 min.

D. Fragment Analysis. The precipitates are dissolved in denaturing gel loading dye (80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue), heat denatured, and electrophoresed in 6% polyacrylamide TBE, 8 M urea denaturing gels. The gels are imaged and analyzed using the STORM™ storage phosphor autoradiography system (Molecular Dynamics, Sunnyvale, CA). Quantitation of protected fragment bands, expressed in femtograms (fg), is achieved by comparing the peak areas obtained from the test samples to those from the known dilutions of the positive control sense strand (see Section B, supra). The results are expressed in molecules of BS322 RNA/cell and as a image rating score. In cases where non-isotopic labels are used, hybrids are transferred from the gels to membranes (nylon or nitrocellulose) by blotting and then analyzed using detection systems that employ streptavidin alkaline phosphatase conjugates and chemiluminesence or chemifluoresence reagents.

Detection of a product comprising a sequence selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof, is indicative of the presence of BS322 mRNA(s), suggesting a diagnosis of a breast tissue disease or condition, such as breast cancer.

## Example 5: Northern Blotting

The Northern blot technique is used to identify a specific size RNA fragment from a complex population of RNA using gel electrophoresis and nucleic acid hybridization. Northern blotting is well-known technique in the art. Briefly, 5-10 µg of total RNA (see Example 3) are incubated in 15 µl of a solution containing 40 mM morphilinopropanesulfonic acid (MOPS) (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, 50% v/v formamide for 15 min at 65°C. The denatured

RNA is mixed with 2 µl of loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) and loaded into a denaturing 1.0% agarose gel containing 40 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA and 2.2 M formaldehyde. The gel is electrophoresed at 60 V for 1.5 hr and rinsed in RNAse free water. RNA is transferred from the gel onto nylon membranes (Brightstar-Plus, Ambion, Inc., Austin, TX) for 1.5 hours using the downward alkaline capillary transfer method (Chomczynski, Anal. Biochem. 201:134-139, 1992). The filter is rinsed with 1X SSC, and RNA is crosslinked to the filter using a Stratalinker™ (Stratagene, Inc., La Jolla, CA) on the autocrosslinking mode and dried for 15 min. The membrane is then placed into a hybridization tube containing 20 ml of preheated prehybridization solution (5X SSC, 50% formamide, 5X Denhardt's solution, 100 µg/ml denatured salmon sperm DNA) and incubated in a 42°C hybridization oven for at least 3 hr. While the blot is prehybridizing, a $^{32}$P-labeled random-primed probe is generated using the BS322 insert fragment (obtained by digesting clone 4304443H1#~~ATCC~~ or another comparable clone with XbaI and NotI) using Random Primer DNA Labeling System (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Half of the probe is boiled for 10 min, quick chilled on ice and added to the hybridization tube. Hybridization is carried out at 42°C for at least 12 hr. The hybridization solution is discarded and the filter is washed in 30 ml of 3X SSC, 0.1% SDS at 42°C for 15 min, followed by 30 ml of 3X SSC, 0.1% SDS at 42°C for 15 min. The filter is wrapped in Saran Wrap, exposed to Kodak XAR-Omat film for 8-96 hr, and the film is developed for analysis. High level of expression of mRNA corresponding to a sequence selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof, is an indication of the presence of BS322 mRNA, suggesting a diagnosis of a breast tissue disease or condition, such as breast cancer.

Example 6: Dot Blot/Slot Blot

Dot and slot blot assays are quick methods to evaluate the presence of a specific nucleic acid sequence in a complex mix of nucleic acid. To perform such assays, up to 50 µg of RNA are mixed in 50 µl of 50% formamide, 7% formaldehyde, 1X SSC, incubated 15 min at 68°C, and then cooled on ice. Then, 100 µl of 20X SSC

are added to the RNA mixture and loaded under vacuum onto a manifold apparatus that has a prepared nitrocellulose or nylon membrane. The membrane is soaked in water, 20X SSC for 1 hour, placed on two sheets of 20X SSC prewet Whatman #3 filter paper, and loaded into a slot blot or dot blot vacuum manifold apparatus. The slot blot is analyzed with probes prepared and labeled as described in Example 4, supra. Detection of mRNA corresponding to a sequence selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof, is an indication of the presence of BS322, suggesting a diagnosis of a breast tissue disease or condition, such as breast cancer.

Other methods and buffers which can be utilized in the methods described in Examples 5 and 6, but not specifically detailed herein, are known in the art and are described in J. Sambrook et al., supra which is incorporated herein by reference.

Example 7: In Situ Hybridization

This method is useful to directly detect specific target nucleic acid sequences in cells using detectable nucleic acid hybridization probes.

Tissues are prepared with cross-linking fixative agents such as paraformaldehyde or glutaraldehyde for maximum cellular RNA retention. See, L. Angerer et al., Methods in Cell Biol. 35:37-71 (1991). Briefly, the tissue is placed in greater than 5 volumes of 1% glutaraldehyde in 50 mM sodium phosphate, pH 7.5 at 4°C for 30 min. The solution is changed with fresh glutaraldehyde solution (1% glutaraldehyde in 50mM sodium phosphate, pH 7.5) for a further 30 min fixing. The fixing solution should have an osmolality of approximately 0.375% NaCl. The tissue is washed once in isotonic NaCl to remove the phosphate.

The fixed tissues then are embedded in paraffin as follows. The tissue is dehydrated though a series of increasing ethanol concentrations for 15 min each: 50% (twice), 70% (twice), 85%, 90% and then 100% (twice). Next, the tissue is soaked in two changes of xylene for 20 min each at room temperature. The tissue is then soaked in two changes of a 1:1 mixture of xylene and paraffin for 20 min each at 60°C; and then in three final changes of paraffin for 15 min each.

Next, the tissue is cut in 5 μm sections using a standard microtome and placed on a slide previously treated with a tissue adhesive such as 3-aminopropyltriethoxysilane.

Many other detection formats exist which can be used and/or modified by those skilled in the art to detect the presence of amplified or non-amplified BS322-derived nucleic acid sequences including, but not limited to, ligase chain reaction (LCR, Abbott Laboratories, Abbott Park, IL); Q-beta replicase (Gene-Trak™, Naperville, Illinois), branched chain reaction (Chiron, Emeryville, CA) and strand displacement assays (Becton Dickinson, Research Triangle Park, NC).

Example 10: Synthetic Peptide Production

Synthetic peptides are modeled and then prepared based upon the predicted amino acid sequence of the BS322 polypeptide consensus sequence (see Example 1). In particular, a number of BS322 peptides derived from SEQ ID NO:24 and SEQ ID NO:25 are prepared, including the peptides of SEQ ID NO:26 and SEQ ID NO:27. All peptides are synthesized on a Symphony Peptide Synthesizer (available from Rainin Instrument Co, Emeryville, CA) or similar instrument, using FMOC chemistry, standard cycles and in-situ HBTU activation. Cleavage and deprotection conditions are as follows: a volume of 2.5 ml of cleavage reagent (77.5% v/v trifluoroacetic acid, 15% v/v ethanedithiol, 2.5% v/v water, 5% v/v thioanisole, 1-2% w/v phenol) is added to the resin, and agitated at room temperature for 2-4 hours. The filtrate is then removed and the peptide is precipitated from the cleavage reagent with cold diethyl ether. Each peptide is filtered, purified via reverse-phase preparative HPLC using a water/acetonitrile/0.1% TFA gradient, and lyophilized. The product is confirmed by mass spectrometry (see Example 12).

Disulfide bond formation is accomplished using auto-oxidation conditions, as follows: the peptide is dissolved in a minimum amount of DMSO (approximately 10 ml) before adding buffer (0.1 M Tris-HCl, pH 6.2) to a concentration of 0.3 - 0.8 mg/ml. The reaction is monitored by HPLC until complete formation of the disulfide bond, followed by reverse-phase preparative HPLC using a water/acetonitrile/0.1% TFA gradient and lyophilization. The product then is confirmed by mass spectrometry (see Example 12).

Example 11b: Expression of Protein in a Cell Line Using pcDNA3.1/Myc-His

A. Construction of a BS322 Expression Plasmid. Plasmid pcDNA3.1/Myc-His (Cat.# V855-20, Invitrogen, Carlsbad, CA) has been constructed, in the past, for the expression of secreted antigens by most mammalian cell lines. Expressed protein inserts are fused to a myc-his peptide tag. The myc-his tag (SEQ ID NO:29) comprises a c-myc oncoprotein epitope and a polyhistidine sequence which are useful for the purification of an expressed fusion protein by using either anti-myc or anti-his affinity columns, or metalloprotein binding columns.

Plasmids for the expression of secretable BS322 proteins are constructed by inserting a BS322 polynucleotide sequence selected from the group consisting of SEQ ID NO:1-9, and fragments or complements thereof. Prior to construction of a BS322 expression plasmid, the BS322 cDNA sequence is first cloned into a pCR®-Blunt vector as follows:

The BS322 cDNA fragment is generated by PCR using standard procedures. For example, PCR is performed procedures and reagents from Stratagene®, Inc. (La Jolla, CA), as directed by the manufacturer. PCR primers are used at a final concentration of 0.5 μM. PCR using 5 U of pfu polymerase (Stratagene, La Jolla, CA) is performed on the BS322 plasmid template (see Example 2) in a 50 μl reaction for 30 cycles (94°C, 1 min; 65°C, 1.5 min; 72°C, 3 min) followed by an extension cycle of 72°C for 8 min. (The sense PCR primer sequence comprises nucleotides which are either complementary to the pINCY vector directly upstream of the BS322 gene insert or which incorporate a 5' EcoRI restriction site, an adjacent downstream protein translation consensus initiator, and a 3' nucleic acid sequence which is the same sense as the 5'-most end of the BS322 cDNA insert. The antisense PCR primer incorporates a 5' NotI restriction sequence and a sequence complementary to the 3' end of the BS322 cDNA insert just upstream of the 3'-most, in-frame stop codon.) Five microliters (5 μl) of the resulting blunted-ended PCR product are ligated into 25 ng of linearized pCR®-Blunt vector (Invitrogen, Carlsbad, CA) interrupting the lethal ccdB gene of the vector. The resulting ligated vector is transformed into TOP10 E. coli (Invitrogen, Carlsbad, CA) using a One Shot™ Transformation Kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The transformed cells are grown on LB-Kan (50 μg/ml kanamycin) selection plates at 37°C. Only cells

tissue. The method utilizes insertion of the gene sequence into a plasmid which contains a CMV promoter, expansion and purification of the plasmid and injection of the plasmid DNA into the muscle tissue of an animal. Preferred animals include mice and rabbits. See, for example, H. Davis et al., Human Molecular Genetics 2:1847-1851 (1993). After one or two booster immunizations, the animal can then be bled, ascites fluid collected, or the animal's spleen can be harvested for production of hybridomas.

B. Plasmid Preparation and Purification. BS322 cDNA sequences are generated from the BS322 cDNA-containing vector using appropriate PCR primers containing suitable 5' restriction sites following the procedures described in Example 11. The PCR product is cut with appropriate restriction enzymes and inserted into a vector which contains the CMV promoter (for example, pRc/CMV or pcDNA3 vectors from Invitrogen, San Diego, CA). This plasmid then is expanded in the appropriate bacterial strain and purified from the cell lysate using a CsCl gradient or a Qiagen plasmid DNA purification column. All these techniques are familiar to one of ordinary skill in the art of molecular biology.

C. Immunization Protocol. Anesthetized animals are immunized intramuscularly with 0.1-100 μg of the purified plasmid diluted in PBS or other DNA uptake enhancers (Cardiotoxin, 25% sucrose). See, for example, H. Davis et al., Human Gene Therapy 4:733-740 (1993); and P. W. Wolff et al., Biotechniques 11:474-485 (1991). One to two booster injections are given at monthly intervals.

D. Testing and Use of Antiserum. Animals are bled and the resultant sera tested for antibody using peptides synthesized from the known gene sequence (see Example 16) using techniques known in the art, such as Western blotting or EIA techniques. Antisera produced by this method can then be used to detect the presence of the antigen in a patient's tissue or cell extract or in a patient's serum by ELISA or Western blotting techniques, such as those described in Examples 15 through 18.

## Example 14: Production of Antibodies Against BS322

A. Production of Polyclonal Antisera. Antiserum against BS322 is prepared by injecting appropriate animals with peptides whose sequences are derived from that of the predicted amino acid sequence of the BS322 nucleotide consensus sequence (SEQ ID NO:9). The synthesis of peptides, SEQ ID NO:26 and

SEQ ID NO:27, is described in Example 10. Peptides used as immunogen either can be conjugated to a carrier such as keyhole limpet hemocyanine (KLH), prepared as described hereinbelow, or unconjugated (i.e., not conjugated to a carrier such as KLH).

1. Peptide Conjugation. Peptide is conjugated to maleimide activated keyhole limpet hemocyanine (KLH, commercially available as Imject®, available from Pierce Chemical Company, Rockford, IL). Imject® contains about 250 moles of reactive maleimide groups per mole of hemocyanine. The activated KLH is dissolved in phosphate buffered saline (PBS, pH 8.4) at a concentration of about 7.7 mg/ml. The peptide is conjugated through cysteines occurring in the peptide sequence, or to a cysteine previously added to the synthesized peptide in order to provide a point of attachment. The peptide is dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Company, St. Louis, MO) and reacted with the activated KLH at a mole ratio of about 1.5 moles of peptide per mole of reactive maleimide attached to the KLH. A procedure for the conjugation of peptide (SEQ ID NO:26) is provided hereinbelow. It is known to the ordinary artisan that the amounts, times and conditions of such a procedure can be varied to optimize peptide conjugation.

The conjugation reaction described hereinbelow is based on obtaining 3 mg of KLH peptide conjugate ("conjugated peptide"), which contains about 0.77 μmoles of reactive maleimide groups. This quantity of peptide conjugate usually is adequate for one primary injection and four booster injections for production of polyclonal antisera in a rabbit. Briefly, peptide (SEQ ID NO:26) is dissolved in DMSO at a concentration of 1.16 μmoles/100 μl of DMSO. One hundred microliters (100 μl) of the DMSO solution are added to 380 μl of the activated KLH solution prepared as described hereinabove, and 20 μl of PBS (pH 8.4) are added to bring the volume to 500 μl. The reaction is incubated overnight at room temperature with stirring. The extent of reaction is determined by measuring the amount of unreacted thiol in the reaction mixture. The difference between the starting concentration of thiol and the final concentration is assumed to be the concentration of peptide which has coupled to the activated KLH. The amount of remaining thiol is measured using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), Pierce Chemical Company, Rockford, IL). Cysteine standards are made at a concentration of 0, 0.1, 0.5, 2, 5 and 20 mM by

dissolving 35 mg of cysteine HCl (Pierce Chemical Company, Rockford, IL) in 10 ml of PBS (pH 7.2) and diluting the stock solution to the desired concentration(s). The photometric determination of the concentration of thiol is accomplished by placing 200 µl of PBS (pH 8.4) in each well of an Immulon 2® microwell plate (Dynex Technologies, Chantilly, VA). Next, 10 µl of standard or reaction mixture is added to each well. Finally, 20 µl of Ellman's reagent at a concentration of 1 mg/ml in PBS (pH 8.4) is added to each well. The wells are incubated for 10 minutes at room temperature, and the absorbance of all wells is read at 415 nm with a microplate reader (such as the BioRad Model 3550, BioRad, Richmond, CA). The absorbance of the standards is used to construct a standard curve and the thiol concentration of the reaction mixture is determined from the standard curve. A decrease in the concentration of free thiol is indicative of a successful conjugation reaction. Unreacted peptide is removed by dialysis against PBS (pH 7.2) at room temperature for 6 hours. The conjugate is stored at 2-8°C if it is to be used immediately; otherwise, it is stored at -20°C or colder.

2. Animal Immunization. Female white New Zealand rabbits weighing 2 kg or more are used for raising polyclonal antiserum. One animal was immunized per unconjugated or conjugated peptide (prepared as described hereinabove). One week prior to the first immunization, 5 to 10 ml of blood is obtained from the animal to serve as a non-immune prebleed sample.

The peptides, SEQ ID NO:26 (conjugated and unconjugated) and SEQ ID NO:28 (unconjugated) were used to prepare the primary immunogen by emulsifying 0.5 ml of the peptide at a concentration of 2 mg/ml in PBS (pH 7.2) which contains 0.5 ml of complete Freund's adjuvant (CFA) (Difco, Detroit, MI). The immunogen is injected into several sites of the animal via subcutaneous, intraperitoneal, and/or intramuscular routes of administration. Four weeks following the primary immunization, a booster immunization was administered. The immunogen used for the booster immunization dose is prepared by emulsifying 0.5 ml of the same unconjugated or conjugated peptide used for the primary immunogen, except that the peptide now was diluted to 1 mg/ml with 0.5 ml of incomplete Freund's adjuvant (IFA) (Difco, Detroit, MI). Again, the booster dose is administered into several sites and can utilize subcutaneous, intraperitoneal and intramuscular

types of injections. The animal is bled (5 ml) two weeks after the booster immunization and the serum is tested for immunoreactivity to the peptide, as described below. The booster and bleed schedule is repeated at 4 week intervals until an adequate titer is obtained. The titer or concentration of antiserum is determined by microtiter EIA as described in Example 17, below. An antibody titer of 1:500 or greater was considered an adequate titer for further use and study.

Table 1. Titer of rabbit anti-BS322 peptide antisera (11 week bleed)

| Peptide Immunogen | Titer |
|---|---|
| SEQ ID NO:26 (conjugated) | >62,500 |
| SEQ ID NO:26 (unconjugated) | >62,500 |
| SEQ ID NO:28 (unconjugated) | 30,600 |

B. Production of Monoclonal Antibody.

1. Immunization Protocol. Mice are immunized using immunogens prepared as described hereinabove, except that the amount of the unconjugated or conjugated peptide for monoclonal antibody production in mice is one-tenth the amount used to produce polyclonal antisera in rabbits. Thus, the primary immunogen consists of 100 μg of unconjugated or conjugated peptide in 0.1 ml of CFA emulsion; while the immunogen used for booster immunizations consists of 50 μg of unconjugated or conjugated peptide in 0.1 ml of IFA. Hybridomas for the generation of monoclonal antibodies are prepared and screened using standard techniques. The methods used for monoclonal antibody development follow procedures known in the art such as those detailed in Kohler and Milstein, Nature 256:494 (1975) and reviewed in J.G.R. Hurrel, ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL (1982). Another method of monoclonal antibody development which is based on the Kohler and Milstein method is that of L.T. Mimms et al., Virology 176:604-619 (1990), which is incorporated herein by reference.

The immunization regimen (per mouse) consists of a primary immunization with additional booster immunizations. The primary immunogen used for the primary

BS322 antigen, and incubated for a time and under conditions sufficient to form labeled BS322 peptide (or labeled protein) / bound antibody complexes and/or patient BS322 antigen / bound antibody complexes. The BS322 antigen in the test sample competes with the labeled BS322 peptide (or BS322 protein) for binding sites on the microparticle. BS322 antigen in the test sample results in a lowered binding of labeled peptide and antibody coated microparticles in the assay since antigen in the test sample and the BS322 peptide or BS322 protein compete for antibody binding sites. A lowered signal (compared to a control) indicates the presence of BS322 antigen in the test sample. The presence of BS322 antigen suggests the diagnosis of a breast disease or condition, such as breast cancer.

The BS322 polynucleotides and the proteins encoded thereby which are provided and discussed hereinabove are useful as markers of breast tissue disease, especially breast cancer. Tests based upon the appearance of this marker in a test sample such as blood, plasma or serum can provide low cost, non-invasive, diagnostic information to aid the physician to make a diagnosis of cancer, to help select a therapy protocol, or to monitor the success of a chosen therapy. This marker may appear in readily accessible body fluids such as blood, urine or stool as antigens derived from the diseased tissue which are detectable by immunological methods. This marker may be elevated in a disease state, altered in a disease state, or be a normal protein of the breast which appears in an inappropriate body compartment.

Example 20: Immunohistochemical Detection of BS322 Protein

Antiserum against a BS322 synthetic peptide derived from the consensus peptide sequences (SEQ ID NO:24 and SEQ ID NO:25) described in Example 14, above, is used to immunohistochemically stain a variety of normal and diseased tissues using standard proceedures. Briefly, frozen blocks of tissue are cut into 6 micron sections, and placed on microscope slides. After fixation in cold acetone, the sections are dried at room temperature, then washed with phosphate buffered saline and blocked. The slides are incubated with the antiserum against a synthetic peptide derived from the consensus BS322 peptide sequences (SEQ ID NO:24 and SEQ ID NO:25) at a dilution of 1:500, washed, incubated with biotinylated goat anti-rabbit antibody, washed again, and incubated with avidin labeled with horseradish peroxidase. After a final wash, the slides are incubated with